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Pyramiding taro cystatin and fungal chitinase genes driven by a synthetic promoter enhances resistance in tomato to root-knot nematode *Meloidogyne incognita*

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ABSTRACT

Meloidogyne incognita, one of the major root-knot nematode (RKN) species in agriculture, attacks many plant species, causing severe economic losses. Genetic engineering of plants with defense-responsive genes has been demonstrated to control RKN. These studies, however, focused on controlling RKN at certain growth stages. In the present study, a dual gene overexpression system, utilizing a plant *cysteine proteinase inhibitor* (*CeCPI*) and a fungal *chitinase* (*PjCHI-1*), was used to transform tomato (*Solanum lycopersicum*) in order to provide protection from all growth stages of RKN. A synthetic promoter, *pMSPOA*, containing NOS-like and SP8a elements, was employed to drive the expression of introduced genes. Gall formation and the proportion of female nematodes in the population, as well as effects on the reproduction of RKN, were monitored in both transgenic and control plants. RKN eggs collected from transgenic plants displayed reduced chitin content and retardation in embryogenesis. The results demonstrated that transgenic plants had inhibitory effects on RKN that were superior to plants transformed with a single gene. The pyramiding expression system produced synergistic effects by the two defense-responsive genes, leading to a detrimental effect on all growth stages of RKN.

1. Introduction

Parasitic nematodes attack, feed on, and colonize lots of flowering plant species, causing severe economic losses worldwide [1]. One of the major genera of parasitic nematodes is the root-knot nematode (RKN), *Meloidogyne*, and the most widespread species in this genus is *Meloidogyne incognita*. Although RKN is a sedentary parasite, in its mobile second-stage (J2) juveniles can penetrate the roots and establish permanent feeding sites in root cells. Juveniles stay in the feeding sites and take in nutrients from the plant tissues. After undergoing three molts, juveniles develop into adults and undergo an accompanying change in morphology [2]. The immobile females have a saccate shape and lay eggs on the surface of roots, whereas the fusiform shaped males migrate out of the roots [3,4]. Large numbers of knots (galls) are visible of the roots of RKN-colonized plants, which interfere with the uptake of nutrients and water by the root system [5]. The shortage of nutrients and water subsequently reduces the growth (including reproductive output) and overall health of the plant, increasing their sensitivity to other diseases [6].

Several strategies have been used to control nematode infections in agricultural production systems. Although the use of chemical nematacides is effective, their use results in environmental pollution and the nematicides have a high level of toxicity to humans [7]. One of the alternatives to the use of synthetic chemicals is biological control which utilizes nematophagous fungi that can infect and destroy nematodes or their eggs [8]. Only a few of nematophagous fungi, however, have been commercialized. The efficacy of nematophagous fungi has been reported to be heavily impacted by environment conditions, as well as the species of nematode present and the species of nematophagous fungi utilized. Such attenuating factors include temperature, moisture, the density of nematodes, and the growth and multiplication of the nematophagous isolate [9]. Another alternative approach

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is represented by the production of RKN-resistant cultivars *via* genetic engineering. Several genes have been demonstrated to enhance the ability of various plant species to withstand RKN colonization and injury [10–12].

Serine and cysteine proteinases have been identified as the major enzymes that function in the digestion of nutrients in nematodes [13]. For this reason, genes whose products inhibit these two proteinases have been used for the genetic engineering of nematode-resistant plants. Cai et al. reported that overexpression of a serine proteinase inhibitor gene (*SpTI-1*), isolated from sweet potato, in hairy roots of sugar beet conferred a high level of resistance to the beet cyst nematode, *Heterodera schachtii* [14]. Another approach to limit nutrient uptake in nematodes is the use of plant cysteine proteinase inhibitors (phytocystatins), which have been identified in various plant species, including taro [15] and potato [16]. Several phytocystatins have been demonstrated to be involved in nematode defense mechanisms, including Oc-I, isolated from rice [17], CeCPI from taro [12], and CCII from maize [18]. Overexpression of *Oc-I* or its variant, *Oc-IΔD86*, in transgenic plants conferred resistance to *Globodera pallida* [17], *H. schachtii*, and *M. incognita* [19]. Similar results were observed when maize cystatin was overexpressed in transgenic banana, resulting in a high level of resistance to *Radopholus similis* [18]. Our previous research demonstrated that *CeCPI* overexpressed in transgenic tomato also reduced the proportion of female nematodes and decreased the production of galls and egg masses after RKN infection [12].

In addition to limiting nutrient uptake, another strategy for genetically engineering RKN resistance in plants is the production of products that lead to the destruction of eggs, thus reducing the number of offspring. Bird and McClure reported that the nematode eggshell is comprised of an outermost vitelline layer, a middle chitinous layer composed of a protein matrix embedded with chitin microfibrils, and an inner lipid layer [20]. The lipid layer provides impermeability to the eggshell, the chitinous layer protects the lipid layer and provides structural strength to the eggshell, and the vitelline layer serves to support the structural uniformity of the egg [21]. A number of nematode-egg, -parasitic fungi have been identified, which secrete chitinases to degrade the chitin microfibrils in the chitinous layer of the eggshells [22–24]. Tikhonov et al. reported that CHI43, a chitinase isolated from *Verticillium suchlasporium*, degraded the eggshells of *G. pallida* [24]. Likewise, Khan et al. demonstrated that the eggs of *Meloidogyne javanica* exhibited large holes in the chitinous layer and a split in the vitelline layer after they were exposed *in vitro* to a chitinase from the fungus, *Paecilomyces lilacinus* [22]. A chitinase gene, *PjCHI-1*, isolated from *Paecilomyces javanicus*, was the first fungal chitinase gene overexpressed in transgenic tomato plants that was demonstrated to confer resistance to RKN infection [11]. Transgenic plants overexpressing *PjCHI-1* suppressed the reproduction of RKN by degrading the chitin layer in eggshells and retarding embryogenesis [11].

We previously reported that a natural promoter, *pSPOA*, obtained from the upstream regulatory sequence regulating tuber-specific sporamin gene expression in sweet potato, produced a rapid and high-level of expression of transgenes in response to wounding and to phytopathogen challenge [25,26]. Several *cis*-acting elements were identified in *pSPOA*, including a wound-response NOS-like element and an SP8a element [27]. Two copies of the NOS-like element were combined with one copy of the SP8a element and a fragment of the *CaMV35S* promoter to generate a synthetic promoter, named as *pMSPOA*. The *GUS* reporter gene, driven by the *pMSPOA* promoter, displayed wound-inducible expression in transgenic *Arabidopsis* [27]. Additionally, defense-responsive genes possess anti-pathogen activity driven by *pMSPOA* promoter displayed different patterns of over-expression in transgenic tobacco [28]. While the expression profile of *pMSPOA* in transgenic *Arabidopsis* was different from what in tobacco, expression patterns using this synthetic promoter have not yet been characterized in tomato.

Delivery of multiple genes into a plant has been demonstrated to be a superior strategy for genetically engineering resistance to phytopathogens [29–31]. Zhao et al. reported that co-expression of two *Bacillus thuringiensis* toxins in transgenic broccoli plants resulted in a high level of resistance to the diamondback moth (*Plutella xylostella*) [31]. Abdeen et al. co-introduced two proteinase inhibitors, potato PI-II and carboxypeptidase inhibitor PCI, into tomato and demonstrated that transgenic plants displayed increased resistance to two insects, *Heliothis obsoleta* and *Liriomyza trifolii* [29]. Similarly, Narusaka et al. reported that the introduction of dual R proteins, RRS1 and RPS4, into transgenic Brassica crops conferred the resistance to fungus *Colletotrichum higginsianum* [32] as well as bacterial wilt, *Ralstonia solanacearum* [33]. Additionally, Quilis et al. indicated that transgenic expression of two proteinase inhibitors, including maize proteinase inhibitor (MPI) and potato carboxypeptidase inhibitor (PCI), in rice displayed high resistance to insect *Chilo suppressalis* and fungus *Magnaporthe oryzae* [34]. The pyramiding gene strategy has also been applied to the genetic engineering of nematode-resistant plants. Urwin et al. delivered a stacked gene construct, that included a cowpea trypsin inhibitor (*CpTI*) and a cystatin (*Oc-IΔD86*), into *Arabidopsis*, and indicated that the resulting transgenic plants displayed enhanced resistance to *H. schachtii* [30]. Although gene stacking is not a new approach, the incorporation of plant cystatin and fungal chitinase in one transgenic plant to provide resistance against root knot nematode has not been attempted. Therefore, the objectives of the present study were to co-deliver *CeCPI* and *PjCHI-1* genes driven by a synthetic promoter (*pMSPOA*) into tomato, and to evaluate the capacity of the resulting transgenic plants to withstand RKN infection. Results indicated that the transgenic tomato plants harboring the pyramiding genes exhibited inhibitory effects on all growth stages of RKN and not only interfered with the development of RKN by reducing egg mass production but also degraded the structure of eggshells and retarded embryogenesis.

2. Materials and methods

2.1. Plant materials

Tomato (*Solanum lycopersicum*) cv. CLN2468D, characterized by heat tolerance and nematode susceptibility, was used in the present study. Seeds were kindly provided by AVRDC – The World Vegetable Center, Tainan, Taiwan. Seeds were surface sterilized using the previously described protocol [11], and germinated on Murashige and Skoog (MS) basal medium under a 16 h photoperiod (54 μmol/s/m^2) at 22 °C.

2.2. Plasmid construction and transformation

The synthetic promoter, *pMSPOA*, was excised from pGEMTeasy/*pMSPOA* as a *Hind*III and *Xba*I fragment and subcloned into pCAMBIA1300 to obtain pCAMBIA1300/*pMSPOA*. The *pMSPOA* fragment was also subcloned into pUC18/*PjCHI-1*-nos [11] to form pUC18/*pMSPOA-PjCHI-1*-nos. The cassette including *CeCPI* and nos poly(A) was excised from pUC18/*CeCPI*-nos [12] as a *Xba*I and *Kpn*I fragment and cloned into pCAMBIA1300/*pMSPOA* to create pCAMBIA1300/*pMSPOA-CeCPI*-nos. The cassette, including *pMSPOA* promoter, *PjCHI-1*, and nos poly(A) was excised from pUC18/*pMSPOA-PjCHI-1*-nos and cloned into *Kpn*I and klenow-filled *Eco*RI restriction sites of pCAMBIA1300/*pMSPOA-CeCPI*-nos to generate the stacked gene construct designated as pMSPOA::*CeCPI*-pMSPOA::*PjCHI-1* (Fig. 1a). The DNA was transformed into

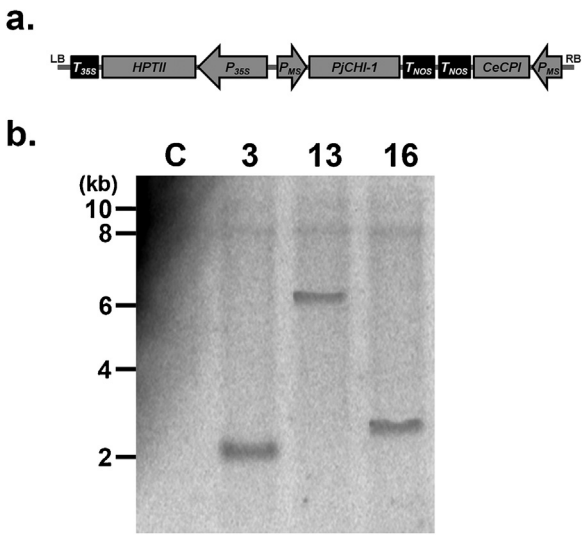

Fig. 1. Identification of T-DNA insertion in the genome of transgenic tomato. (a) Schematic diagram of the expression cassette of the binary vector pMSPOA::*CeCPI*-pMSPOA::*PjCHI-1*. P_{35S}, cauliflower mosaic virus *35S* promoter; P_{MS}, a synthetic *pMSPOA* promoter; *HPTII*, hygromycin phosphotransferase II cDNA; *PjCHI-1*, *Paecilomyces javanicus* chitinase cDNA; *CeCPI*, taro *cystatin* cDNA; T_{35S}, cauliflower mosaic virus 35S terminator sequence; nos, nopaline synthase terminator sequence; LB, left border; and RB, right border. (b) Southern blot analysis of transgenic tomato. Genomic DNA isolated from untransformed control plants (C) and transgenic lines (3, 13, and 16) of tomato (lines 3, 13, and 16) hybridized with a probe for *HPTII*.

the CLN2468D tomato cultivar *via* *Agrobacterium*-mediated transformation [35].

2.3. Molecular characterization of transgenic tomato plants

Genomic DNA of T_1 transgenic plants was extracted with a buffer containing 1.4 M NaCl, 0.2% β-mercaptoethanol, 0.1 M Tris–HCl, pH 8.0, 0.02 M EDTA, pH 8.0, and 2% hexadecyltrimethylammonium bromide. After treatment with RNaseA and precipitation with isopropanol, the DNA was then subjected to Southern blot hybridization to detect the number of foreign gene insertions [32]. Fifteen μg of DNA was digested with 50 U of *Eco*RV, and fractionated on a 0.8% agarose gel. The DNA was then blotted onto an Immobilon Hybond N^+ membrane in 20× sodium chloride/sodium citrate (SSC) buffer and UV cross-linked. The full length of the hygromycin-resistant gene *HPTII* was amplified by PCR and labeled with (α-^{32}P) dCTP using the *redi*prime labeling system (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's protocol. The α-^{32}P-dCTP-labeling probe was subjected to the hybridization reaction with the membranes at 42 °C. After stringent washing, the membrane was visualized using autoradiography.

To detect the expression of transgenes, total RNA was extracted from the roots of transgenic plants. Root samples were ground in liquid nitrogen and total RNA was isolated using an extraction buffer containing 2% polyvinylpyrrolidone K30, 2% hexadecyltrimethylammonium bromide, 25 mM EDTA, 0.05% spermidine, 2 M NaCl, 0.1% diethyl pyrocarbonate (DEPC), 100 mM Tris–HCl, pH 8.0, and 2% β-mercaptoethanol. RNA was then precipitated with 10 M LiCl at 4 °C for 16 h, and re-suspended in DEPC-treated water. For reverse transcription-polymerase chain reaction (RT-PCR) analysis, 1 μg of total RNA was mixed with 200 nM poly-T and incubated at 65 °C for 5 min. The mixture was then mixed with 5× reaction buffer (Fermentas), 200 μM dNTP mixture, 200 U of RevertAid™ M-MLV reverse transcriptase (Fermentas), Ribolock™ RNase inhibitor and incubated at 42 °C for 1 h. The RT product was amplified using primers designed to amplify full-length *PjCHI-1* and *CeCPI*. The primers used for *CeCPI* were 5′-TCTAGAATGGCCTTGATGGGGGGC-3′ located at the 5′ end of *CeCPI*, and 5′-TCGCAAGACCGGCAACAGGATTC-3′ located at the 5′ end of nos poly (A). The primers used for *PjCHI-1* were 5′-TCTAGAATGACCGATGAGTGGGCCGATACT-3′, located at the 5′ end of *PjCHI-1*, and the primer located at the 5′ end of nos poly(A) described above. The tomato *Ubiquitin3* gene was amplified as an internal control.

2.4. Cysteine proteinase activity assay

Total protein was extracted from the roots of untransformed CLN2468D and transgenic plants, and *in vitro* cysteine proteinase activity was assayed as described by Beyene et al. [36]. Total protein was extracted with 50 mM Tris–HCl, pH 7.4. After estimating protein concentration, the total protein extract was mixed with a proteolysis buffer containing 100 mM citrate phosphate, pH 6.0 and 10 m L-cysteine at 37 °C for 10 min. Subsequently, the mixture was mixed with proteinase substrate, 20 μM Z-phe-arg-AMC, at 37 °C for 10 min. A stopping solution containing 10 mM sodium monochloroacetate, 30 mM sodium acetate and 70 mM acetic acid was then added into the mixture. Fluorescence generated by AMC was analyzed on a fluorescence microplate reader (Labsystems Fluoroskan Ascent FL, Type 374, Thermo Scientific, MA, USA) using an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

2.5. Endochitinase activity analysis

To analyze the endochitinase activity of root tissues, total protein was extracted as previously described [11]. To quantify the activity, 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside (Sigma M5639) was utilized as a substrate according to the procedure previously reported [11]. The endochitinase activity was estimated as picomoles of 4-MU generated per minute per microgram of total protein.

2.6. Nematode assay

Transgenic tomato seedlings were identified by screening for hygromycin resistance, while untransformed plants were grown on MS medium only. After incubation at 25 °C for 3 weeks, seedlings were transplanted to sandy soil and acclimated for 2 weeks. The preparation of RKN inoculum and the infection of RKN on test plants were carried out as previously described [12]. To analyze the resistance of transgenic plants to RKN infection, the penetration efficiency of RKN, the quantity of galls and egg mass, and the growth and development of nematodes were assayed as previously described [12].

To evaluate the integrity of RKN eggs, egg masses were collected from the roots of control and transgenic plants. Several parameters, including the chitin content in eggshells and the development of embryos in eggs were analyzed as previously described [11]. An *in vitro* endochitinase activity was performed on egg masses collected from control and transgenic plants as described above.

2.7. Statistical analysis

The effect of the transgenic plants on various parameters was determined using ANOVA. Data are presented as the mean ± SD. Separation of means was determined using a Scheffe's test at 5%, 1% and 0.1% probability levels.

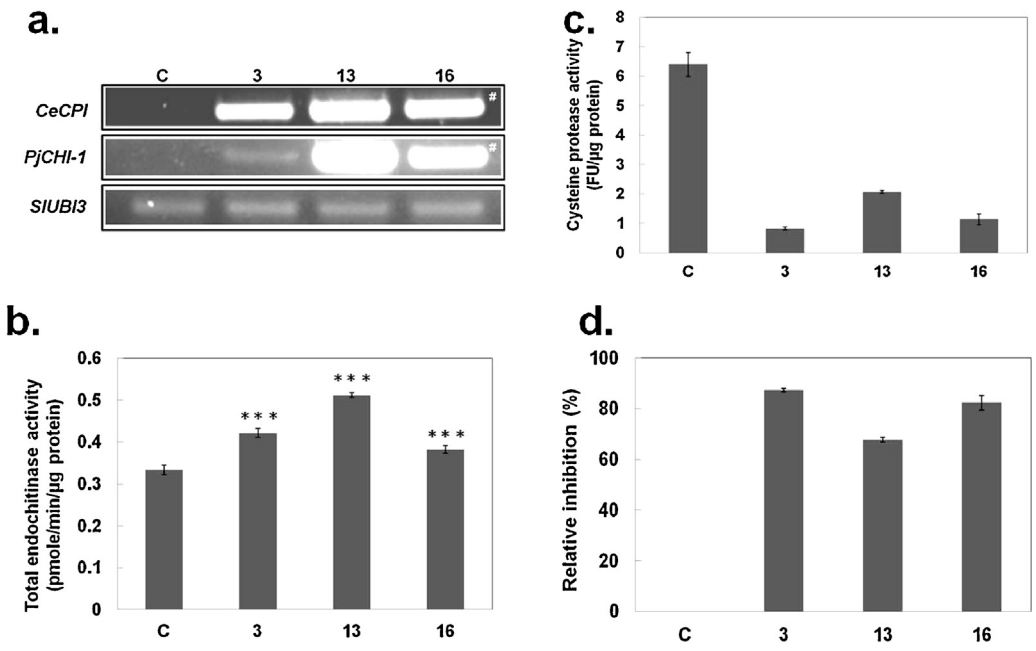

Fig. 2. Overexpression of dual transgenes in transgenic tomato plants. (a) RT-PCR amplification of *CeCPI* and *PjCHI-1* transcripts in transgenic tomato. Total RNA in each sample was amplified using primers for *CeCPI* and *PjCHI-1*. Tomato *Ubiquitin3* was used to ensure even loading of DNA. # indicates a 10-fold dilution of *CeCPI* and *PjCHI-1* transcripts in line 16 for agarose gel electrophoresis. (b) Endochitinase activity in control and transgenic tomato plants was analyzed in triplicate. Data are means ± SD. ***, significant difference at $P < 0.001$. (c) Cysteine proteinase activity in control and transgenic tomato was detected using a fluorescence assay. (d) The inhibition of cysteine proteinase activity in transgenic plants relative to the activity in control plants was calculated in order to document the function of CeCPI.

3. Results

3.1. Overexpression of CeCPI and PjCHI-1 genes in transgenic tomato

Sixteen independent, putative transgenic tomato lines were generated using an *Agrobacterium*-mediated transformation system, followed by hygromycin selection and regeneration. The putative transgenic plants were positively identified as transformants by PCR amplification of *PjCHI-1* and *CeCPI* (data not shown). No obvious phenotypic differences were observed between wild-type and transgenic plants in relation to their appearance and overall pattern of growth (data not shown). Transgenic lines 3, 13 and 16, characterized by a high seed set were selected for further studies. Southern blot analysis of T_1 transgenic lines indicated one insertion of the hygromycin-resistant gene *HPTII* (Fig. 1b). The highest level of expression of both *PjCHI-1* and *CeCPI* was detected in line 16, while the least was recorded in line 3 (Fig. 2a). The results of the chitinase activity assay, however, were not consistent with the levels of expression of *PjCHI-1* observed in the individual lines. No significant difference in chitinase activity was recorded between line 3 and 16, while the highest activity was detected in line 13 (Fig. 2b). A similar inconsistency was observed between the expression level of *CeCPI* and cysteine proteinase activity. Line 13, whose level of *CeCPI* expression was not the lowest among the three lines (Fig. 2a), exhibited the highest level of cysteine proteinase activity (Fig. 2c) and the lowest level of inhibition of cysteine proteinase (Fig. 2d). Although the transcriptional levels of both genes were not absolutely consistent with the activity levels of the proteins coded by these genes in the individual transgenic lines, T_1 plants of all three transgenic lines displayed high levels of both endochitinase activity and cysteine proteinase inhibitory activity (Fig. 2). In addition, transgenic plants harboring single defense-responsive gene were generated; the detection of transcripts indicated that single transgene highly expressed in transgenic plants (Figs. S1a and S2a). The biochemical activity of the expressed defense proteins in each transgenic tomato plants were also confirmed (Figs. S1b and S2b).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.11.010.

3.2. Reduction in gall numbers in RKN-infected roots of transgenic plants

No significant difference was observed between the root mass in control and T_1 transgenic plants grown on a medium supplemented with hygromycin (Fig. 3a), indicating that the root growth in the transgenic plants was not affected by the transgenes or by hygromycin. Similar number of RKNs was also observed in the roots of transgenic and control plants two days post-infection, suggesting that the transgenes did not interfere with the ability of RKN to invade the root systems in transgenic plants (Fig. 3b). The numbers of galls present in transgenic plants, however, was significantly lower than the number of galls in control plants after RKN-infection (Fig. 3c). All 3 lines had a significantly lower number of galls relative to the control plants, representing about a 50% reduction in overall gall numbers. Although the levels of cysteine proteinase inhibition varied among the three transgenic lines (Fig. 2d), no significant difference in gall numbers was observed between the different transgenic lines (Fig. 3c). Similar results were recorded in the transgenic plants harboring only *CeCPI* gene (Fig. S1c). However, the number of galls was not reduced in most of transgenic plants expressed *PjCHI-1* only (Fig. S2c). Collectively, the data indicate that transgenic tomato plants overexpressing the pyramided *CeCPI* and *PjCHI-1* genes exhibited a dramatic reduction in gall numbers but not in the invasion of nematodes.

3.3. Reduction in the proportion of female RKN in transgenic plants

Fusiform, saccate, and enlarged saccate shapes were observed in the RKN infecting the roots of both control and transgenic plants at six weeks post-infection. The size of RKNs, including male and female RKNs, collected from transgenic plants did not significantly

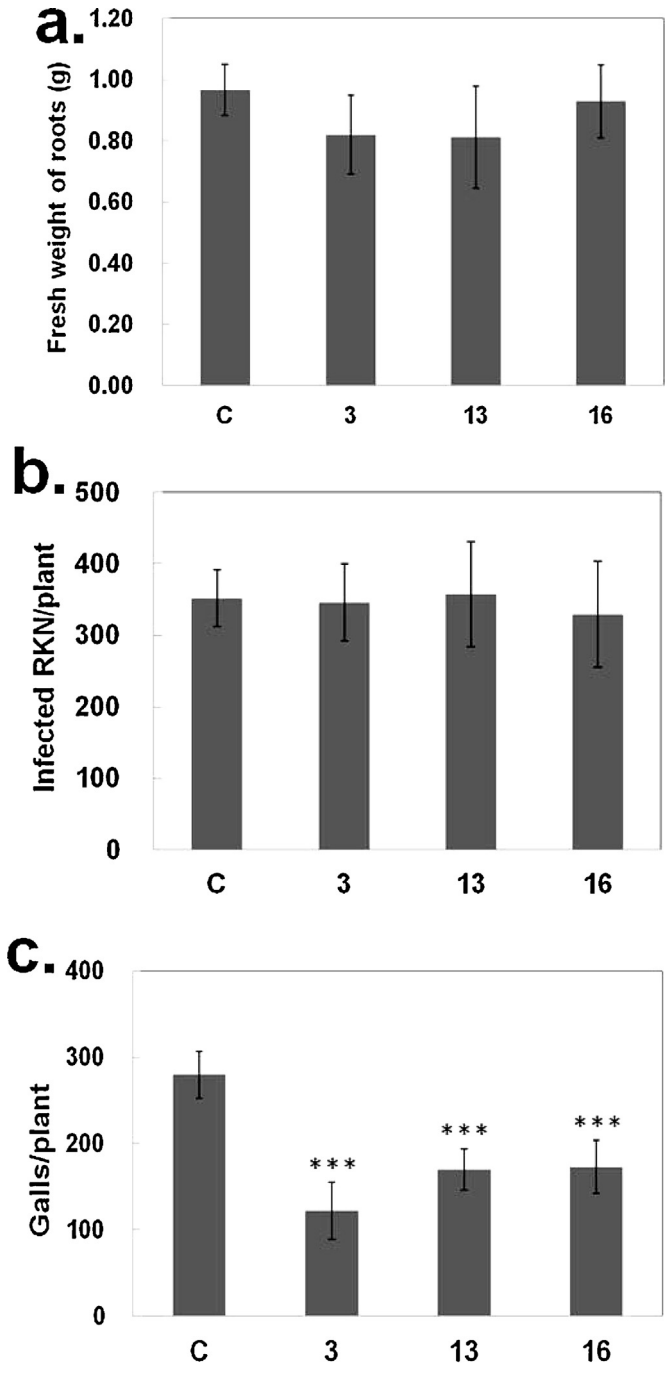

Fig. 3. Reduction in gall formation but not RKN penetration in transgenic plants. (a) The fresh weight of roots of experimental plants was recorded at 3 weeks post-germination on MS medium without hygromycin (C, control) or with hygromycin (transgenic lines 3, 13 and 16). Five replicates were examined from each line. (b) The number of nematodes present inside the roots of the different transgenic lines and the control plants was determined in T_1 plants inoculated with *M. incognita* for 2 days. (c) The total number of galls was counted in control and transgenic T_1 plants post-infection. Data was analyzed by ANOVA. *** indicates a significant difference at $P < 0.001$.

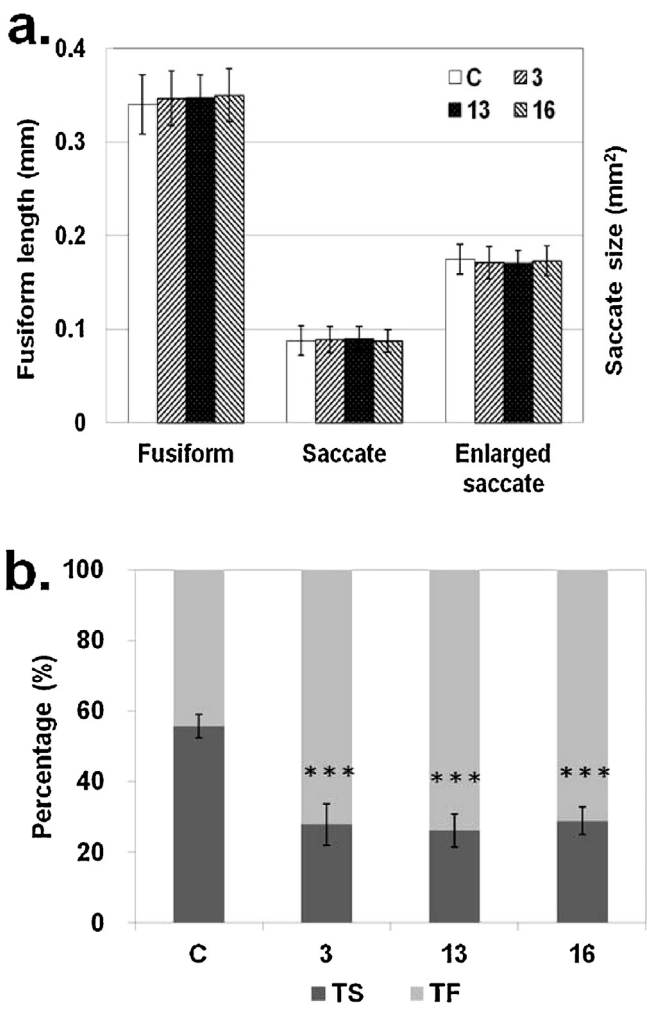

Fig. 4. Reduction in the proportion of female *M. incognita* in transgenic plants post-infection. (a) The growth of nematodes inside the roots of control and transgenic plants was examined. Length of fusiform-shaped RKNs was measured while the overall size of saccate-shaped RKNs was calculated. (b) The proportion of RKNs with a fusiform or a saccate shape was calculated in the different lines of experimental plants. TS represents the percentage of female nematodes with saccate and enlarged saccate shapes in the nematode population, while TF symbolize the percentage of nematodes with a fusiform shape in the nematode population. *** indicates a significant difference at $P < 0.001$.

differ from the size of RKNs isolated from control plants (Fig. 4a), indicating that the growth of RKNs was not affected in transgenic plants. Although the growth of RKNs was not affected, a much lower proportion of female nematodes, those with saccate and enlarged saccate shapes, was observed in transgenic plants compared to the proportion observed in control plants (Fig. 4b). In control plants, 55% of the collected nematodes were female and 45% were male. In comparison, only 29% of the nematodes were female in RKNs collected from transgenic plants (Fig. 4b). These results suggest that although the recombinant proteins in transgenic lines of tomato did not interfere with the growth of nematodes, the proteins had a significant impact on the nematode development.

3.4. Reduction in the number of RKN egg masses produced in transgenic plants

The reduction in the proportion of female nematodes observed in transgenic plants (Fig. 4b) implied that RKN reproduction may have also decreased. Therefore, the abundance of egg masses in control and transgenic plants was recorded after erioglaucin staining (Fig. 5a). Results indicated that the number of egg masses in transgenic lines was reduced to approximately half the number observed in control plants (Fig. 5b). Although the number of egg mass was reduced in transgenic plants harboring only *CeCPI* gene, the magnitude of reduction was not obvious as what was recorded in the plants expressed dual transgenes (Fig. S1d and Fig. 5b). In contrast, no significant repression of egg mass production was recorded in the transgenic plants expressed *PjCHI-1* only (Fig. S2d).

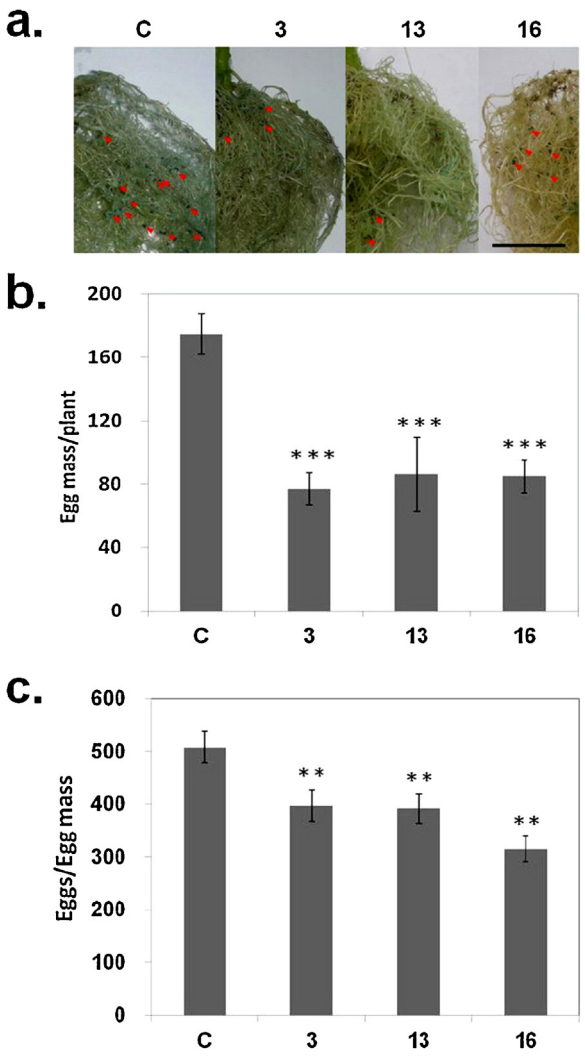

Fig. 5. Suppression of RKN reproduction in transgenic tomato. (a) Egg masses stained with erioglaucin at six weeks post RKN infection were observed in the roots of control and transgenic plants. (b) The number of egg masses in each root system. (c) The number of eggs in each egg mass collected from transgenic and control plants. Data are presented as mean ± SD. ** and *** indicate a significant difference at $P < 0.01$ or $P < 0.001$, respectively.

Additionally, the number of eggs within each egg mass was also significantly lower in transgenic plants expressed dual defense-responsive genes relative to the control plants. The least number of eggs per egg mass was observed in line 16, where the average egg number per egg mass was 314 compared to an average of 507 eggs per egg mass in control plants (Fig. 5c). These results suggest that the production of offspring by female nematodes was significantly impacted in transgenic tomato plants overexpressing dual *CeCPI* and *PjCHI-1*.

3.5. Embryogenesis in RKN eggs is impeded in transgenic plants

Embryos in nematode eggs have been reported to be injured by incubating the eggs in a fungal chitinase *in vitro* [22,37] or by the production of recombinant chitinase proteins in transgenic plants [11]. Therefore, we examined the developing embryos in eggs collected from transgenic and control plants. Less than 25% of the eggs in control plants remained at a single-cell stage, while nearly 40% of the eggs in transgenic lines remained at a single-cell stage (Fig. 6). In contrast, the opposite trend was observed in eggs at the J1 stage (Fig. 6). Less than 35% of the eggs collected from transgenic plants

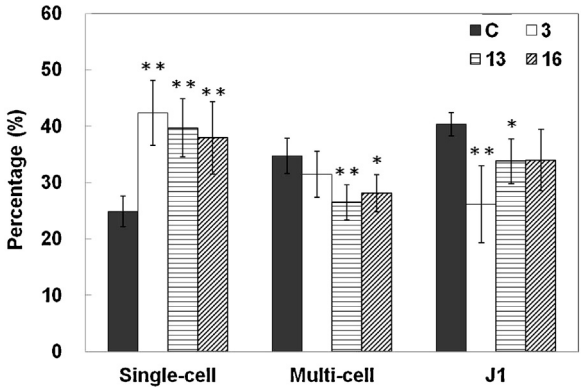

Fig. 6. Retardation of RKN embryonic development in transgenic plants. Eggs collected from control (C) and transgenic plants (lines 3, 13 and 16) were divided into single-cell, multi-cell, and juvenile stages; the percentage of nematodes in each stage in the population was calculated. Data are presented as mean ± SD. * and ** indicate a significant difference at $P < 0.05$ or $P < 0.01$, respectively.

developed into J1 nematodes, compared to about 40% of the eggs in control plants being at the J1 stage. This reduction in the number of J1 nematodes was greatest in line 3. The results indicated that fewer eggs developed into juveniles in the transgenic plants compared to the number in control plants, suggesting that the embryonic development of eggs was greatly impeded in transgenic plants.

3.6. RKN eggshells are damaged in transgenic plants

The integrity of the chitin layer in RKN eggs collected at various stages of development was examined in both control and transgenic plants. Histological staining using lactophenol cotton blue revealed a much stronger blue staining in eggs obtained from control plants at the J1 stage than at the cell-stage, indicating that the amount of chitinous compounds in RKN eggs increase as they develop from the cell-stage to the J1 stage (Fig. 7a). A relatively weak staining of eggshells was observed, however, in both cell and J1 stage eggs collected from the transgenic lines (Fig. 7a). Chitinase activity measured in the egg masses obtained from transgenic plants was 0.2 pmol/min/g protein, while the activity in egg masses from control plants was undetectable (Fig. 7b). These results suggest that the elevated activity of PjCHI-1 may be responsible for the thin and less chitinous layer in the eggs collected from transgenic tomato lines. Additionally, the impeded embryogenesis observed in the eggs collected from transgenic tomato plants (Fig. 6) may be a direct result of the damaged eggshells.

4. Discussion

A stacked gene construct combining a phytocystatin *CeCPI* gene and a fungal chitinase *PjCHI-1* gene, under the control of the *pMSPOA* promoter, was introduced into tomato. Although both genes were constitutively expressed at high levels in all of the transgenic lines, the levels of transcription were not totally consistent with translational levels. Similar results were reported in transgenic tomato expressing *PjCHI-1* driven by *CaMV35S* promoter [11]. Unidentified aspects related to either the random insertion of the construct into the host DNA or post-transcriptional modifications of the transgenes may contribute to the lack on concordance between transcription and translation [38,39]. The transgenic lines of tomato generated in this study, however, did express both transgenes and exhibited resistance to RKN infection, suggesting that the levels of recombinant proteins produced were associated with the observed levels of RKN resistance.

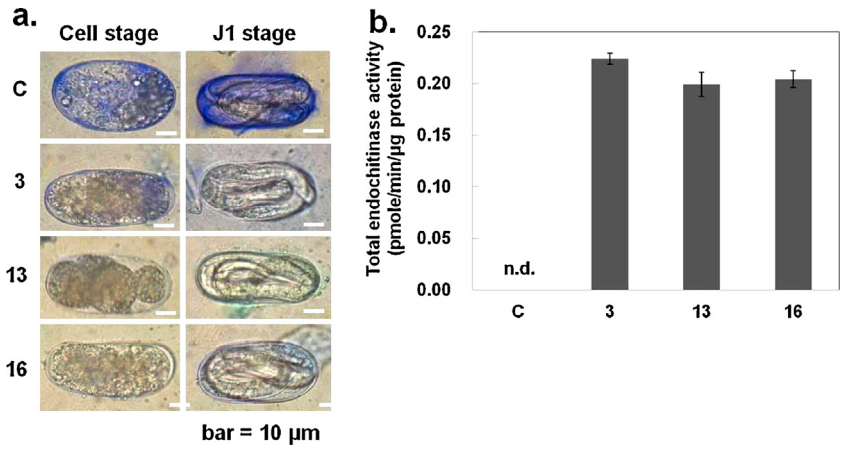

Fig. 7. Reduction in the chitin content of *M. incognita* eggs obtained from transgenic plants. (a) *M. incognita* eggs were collected from control (C) and transgenic plants (lines 3, 13 and 16), and stained with lactophenol cotton blue to evaluate the chitin content of eggshells. The stained eggs were photographed under a stereomicroscope (100× magnification). (b) Endochitinase activity in egg masses obtained from control (C) and transgenic plants (lines 3, 13 and 16). Data are presented as the mean ± SD of three biological replicates ($n = 3$).

Our previous studies demonstrated the role of *CeCPI* and *PjCHI-1* in the defense against RKN [11,12]. Overexpression of *CeCPI* alone resulted in a decrease in the number of galls and egg masses, as well as the ratio of female nematodes in infected roots [12]. PjCHI-1 was observed to negatively impact the reproduction of RKN, including a reduction in the numbers of egg masses and egg numbers per egg mass. The embryonic development in eggs and the chitin layer in eggshells were both detrimentally affected by recombinant PjCHI-1 [11]. In the present study, transgenic plants expressing two transgenes (a phytocystatin *CeCPI* gene and a fungal chitinase *PjCHI-1* gene) exhibited all of the negative impacts on RKN mentioned above, indicating that the transgenic plants displayed dual resistant systems against RKN infection. After penetration into the root tissues of transgenic plants, RKN was first impacted by CeCPI, repressing the formation of galls and the proportion of female nematodes. Subsequently, the reproduction of RKN was further impacted by both CeCPI and PjCHI-1, resulting in a reduced number of egg masses and eggs per egg mass. The eggs on the surface of roots were further impacted by PjCHI-1, resulting in damage to the eggshells and impeded development of the RKN embryos. Importantly, expression of both of the stacked genes in transgenic tomato plants was able to negatively impact all stages of the RKN life cycle, thus providing a more effective protection against RKN than the overexpression of just one of the individual genes.

The synthetic promoter, *pMSPOA*, was utilized in the present study to drive the expression of *CeCPI* and *PjCHI-1*. Although the *pMSPOA* promoter is wound-inducible in transgenic *Arabidopsis* [27], it resulted in constitutive expression in transgenic tomato. Similar results were reported in transgenic tobacco expressing *sporamin* of sweet potato and *CeCPI* when expression was driven by *pMSPOA* [28]. In that study, both transgenes were constitutively expressed rather than exhibiting wound-inducible expression. These contrasting results suggest that the wound-inducible activity of *pMSPOA* may be species specific. While it drives wound-inducible expression in *Arabidopsis*, it results in constitutive expression of transgenes in tomato and tobacco. Changes in promoter activity in transgenic plants have been previously reported [32]. For example, *pRB7* drives root-specific expression in tobacco, the species from which it was originally isolated, but resulted in constitutive expression of transgenes in all tissues of tomato except fruits [32]. Although the expression pattern of genes may change when promoters are introduced into other plant species, the use of promoters with distinctive characteristics, such as inducible or tissue-specific expression, may facilitate the development of desired traits in plants *via* genetic engineering.

Gene stacking strategies have been previously applied in the generation of nematode-resistant cultivars. Urwin et al. reported that a dual proteinase inhibitor construct, consisting of a cowpea trypsin inhibitor, *CpTI*, and a cystatin, *Oc-IΔD86*, enhanced the resistance of transgenic *Arabidopsis* to the cyst nematode, *H. schachtii* [30]. In that study, transgenic plants exhibited a suppression in the growth and number of female nematodes [30], suggesting a synergistic effect of the transgenes. Additionally, a construct that combined a maize kernel cystatin, *CCII*, with a synthetic peptide, nAChRbp, which can interfere with nematode chemoreception, was introduced into banana, and resulted in a high level of resistance in transgenic banana plants to *R. similis* and *H. schachtii* [18]. These studies demonstrate that transgenic plants expressing dual defense-responsive genes not only provide two defense systems against nematodes but also broaden the spectrum of resistance to several nematode species. In the present study, the overexpression of both *CeCPI* and *PjCHI-1* in transgenic tomato resulted in an evident synergistic effect on RKN resistance, enhancing the efficacy of RKN resistance in transgenic plants. Two defense systems resulting from the overlapping impact of both CeCPI and PjCHI-1 on RKNs conferred increased levels of RKN resistance in transgenic tomato plants. One defense system interferes with gall and egg mass production, while the other defense system inhibits embryonic development and the integrity of eggshells. Combining the two defense activities provides resistance to RKN at all stages of its life cycle. Hence, the present study provides a new approach to genetically engineering RKN resistance in tomato plants based on stacking of at least two genes that complement each other and whose expression results in a synergistic action that confers a high level of RKN resistance in transgenic plants.

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